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(57) Abstract

The triester phosphorothioate oligonucleotides disclosed comprise internucleotide concatenations having a P-S' bond protected by a bioreversible grouping (X) in intracellular media.

(57) Abrégé

La présente invention a pour objet des oligonucléotides phosphorothioates triesters caractérisés en ce qu'ils comportent des enchaînements internucléotidiques qui présentent une liaison P-S' protégée par un groupement (X) bioréversible dans les milieux intracellulaires.

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PHOSPHOROTHIOATE TRIESTER OLIGONUCLEOTIDES AND METHOD OF PREPARATION

The present invention relates to phosphorothioate triester oligonucleotide compounds and to a method of
5 preparation.

In the present application, the term "oligo-nucleotides" generally speaking indicates a DNA or RNA polynucleotide, that is to say one in the ribo- (RNA) or deoxyribo- (DNA), or even mixed ribo-deoxyribo, series.
10 These oligonucleotides are in general formed by a linkage of 2 to 100 nucleotides, and more generally, of 5 to 50 nucleotides.

These oligonucleotides are used for biological or therapeutic purposes according to different approaches:
15 antisense (formation of duplex), anti-gene (formation of triple helixes), catalytic (RNA with ribozyme activity) or sense (protein target).

The antisense oligonucleotides are short synthetic DNA or RNA or mixed molecules of complementary
20 sequences to a target sequence belonging to a gene or to an RNA messenger whose expression it is specifically des-ired to block. The antisense oligonucleotides are in fact directed against a messenger RNA sequence, or altern-atively against a DNA sequence and hybridize to the
25 sequence to which they are complementary, thus being able to block genetic expression.

The antisense deoxyribonucleotides can also be directed against certain bicatenary DNA regions (homo-purine/homopyrimidine sequences or sequences rich in
30 purines/pyrimidines) and thus form triple helixes. These oligonucleotides directed in this way against DNA have been called "anti-gene" or alternatively "anti-code". The formation of a triple helix, at a particular sequence, can block the fixing of proteins intervening in the
35 expression of a gene and/or allow irreversible damage to be introduced into the DNA if the oligonucleotide under consideration possesses a particular reactive group. Such antisense oligonucleotides can form artificial restric-tion endonucleases, directed against specific sequences.

In cells, and more particularly in an organism, in the blood circulation, for example, the natural oligonucleotides are sensitive to degradation by nucleases. Nucleases are degradation enzymes capable of

5   cutting the phosphodiester bonds of DNA or of RNA, either by introducing internal cleavages into mono- or bicatenary molecules, or by attacking these molecules starting from their ends. The enzymes which attack internally are called endonucleases and those attacking by the ends are

10   called exonucleases.

      The use of oligonucleotides encounters two major problems which are, on the one hand, great sensitivity to degradation by exonucleases which are found as well in the serum or in extracellular medium or in intracellular

15   cytoplasmic medium and, on the other hand, low intracellular penetration.

      The use of modified oligonucleotides has already been proposed to increase the stability of oligonucleotides or favor penetration through cellular membranes or

20   alternatively to stabilize hybridization and specific affinity for a target sequence, whether this be a single or double strand nucleic acid, or even a protein, or alternatively to increase the interaction with said target sequence. Chemical modifications of the structural

25   skeleton of the molecule or derivations or couplings to reactive or intercalating groups have been proposed, in general localized at the end of the oligonucleotides.

      As far as the chemical modifications of the skeleton are concerned, it has been proposed to modify

30   the nature of the internucleotide phosphate linkage, especially in the form of methylphosphonate, phosphorothioate or phosphorodithioate; or alternatively by modifying the sugar part, especially by an alpha-anomeric configuration, a $2'-O-CH_3$ substitution or by replacing

35   the oxygen of the furan ring by a sulfur (4'-thioribonucleotide). It has also been proposed to modify the nucleotide bases.

      Thus, in French Patent Applications FR 83 01223 (2 540 122) and FR 84 11795 (2 568 254)

chemical compounds formed by an oligonucleotide including natural or modified linkage of β-nucleotides have been described, on which are found, fixed by a covalent bond, at least one intercalating group, which compounds have

5   the property of selectively blocking the expression of a gene and which therefore are particularly useful in therapy as antiviral, antibiotic, antiparasitic or antitumor substances.

       In International Application WO 88/04301, oligo-

10   nucleotides of alpha-anomeric configuration have been described having more stable parallel pairings with complementary sequences.

       However, the uses proposed until now raise other problems, especially of toxicity, as chemical modifica-

15   tions introduced into the molecule prove to be capable of inducing toxicity on the pharmacological level in certain therapeutic applications. Generally speaking, it is difficult to combine the different criteria of resistance to nucleases, stabilization of hybridization, penetration

20   into the cell and, likewise, increase in activity yielding the duplex with the RNAse H substrate complementary target sequence, which has the property of cleaving the DNA/RNA duplexes.

       The oligonucleotide compounds whose phosphate

25   part was modified by methylphosphonate are particularly resistant to degradation by nucleases. However, the electrically neutral oligomers such as the oligomers of the methylphosphonate type penetrate more easily into the cell than the electrically charged oligomers such as the

30   phosphodiesters. However, these methylphosphonate derivatives have a chirality, especially at the phosphate, thus leading to the formation of diastereoisomers. In addition, the RNA/oligodeoxynucleotide methylphosphonate duplexes are not substrates of RNAseH. The oligomer

35   derivatives of the phosphorothioate diester type have a resistance to degradation by nucleases but to a lesser degree than the methylphosphonate oligomers. On the other hand, they lead to electrically charged oligomers capable of activating RNAse H, but penetrate less easily into the

cell than the methylphosphonate oligomers.

Generally speaking, the oligonucleotides which are the subject of the invention are intended to provide novel stable oligonucleotides capable of being internal-
5   ized in cells and capable of hybridizing and/or of having an affinity for specific nucleic acid or protein sequences and thus of interacting with cellular or viral factors.

The present invention provides oligonucleotides
10 with electrically neutral phosphorothioate triester linkages being able to re-form, after penetration into the cell, and selectively, phophorothioate diester or polar phosphodiester bonds. Analogously, the present invention includes phosphorodithioate triester linkages
15 giving, intracellularly, phosphorodithioate diester or phosphorothioate diester linkages.

To do this, P-S⁻ bonds of an oligomer are selec-tively protected by a bioreversible group (X) in the intracellular media.

20 The modifications proposed according to the invention produce oligomers having the advantageous properties of phosphorothioate diester derivatives, while overcoming the disadvantages of the latter, especially as far as their sensitivity to extracellular exonucleases
25 and their difficulty in penetrating through the cellular membrane is concerned.

The present invention thus relates more precisely to a phosphorothioate or phosphorodithioate triester oligonucleotide, characterized in that it comprises
30 internucleotide linkages which have a P-S⁻ bond protected by a bioreversible group (X) in intracellular media.

The route of synthesis used in this invention consists in the use of a nucleophilic substitution reaction by the sulfur atom of the P-S⁻ bond of an alkyl-
35 ating agent XL, L being a leaving group (halogen, ester, tosyl...) and X a bioreversible group. It follows that it is possible to convert the P-S⁻ functions into corresponding bioreversible phosphotriesters, as will be shown in the examples below.

Such a process does not require protection of heterocyclic bases and can therefore be carried out directly on a previously prepared oligonucleotide. It is thus possible to obtain phosphorothioate triester oligomers starting from phosphorothioate diester oligomers. In fact, a mixed linkage comprising phosphodiester (P-O⁻) and phosphorothioate (P-S⁻) bonds will be selectively alkylated at the sulfur atoms.

The phosphorothioate triester oligomers according to the present invention are electrically neutral in the extracellular medium and therefore benefit from improved penetration into the cell. In addition, they allow electrically charged phosphodiester or phosphorothioate diester oligonucleotides to be delivered intracellularly which are capable, as such, of being substrates of RNAse H when they form a mixed RNA/DNA duplex with their target complementary sequences.

The principle of the invention applies to any synthetic oligonucleotide, of the DNA, RNA or mixed series, whatever the biological target envisaged, insofar as it has internucleotide linkages containing a P-S⁻ bond capable of being alkylated by bioreversible groups.

In one embodiment, the oligonucleotides according to the present invention correspond to the formula
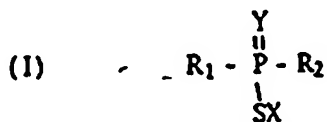
$$(I) \qquad - \; _- R_1 - \overset{\overset{\displaystyle Y}{\parallel}}{\underset{\underset{\displaystyle SX}{\mid}}{P}} - R_2$$

in which:

-    Y is O or S
-    R_1 and R_2 are respectively a residue in the 3'-O and 5'-O positions of a nucleoside or of an oligonucleotide, the internucleotide linkage of which is natural or modified, the linkage of which is especially of the phosphorothioate triester type of formula

$$Y - \overset{\mid}{\underset{\mid}{P}} - SX$$
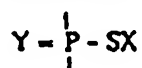
-    X is a $-(CH_2)_n-Y^1-W$ radical or

- X is a $-(CH_2)_n-Y^1-W$ radical or

    · Y^1 is S or O

    · n varies from 1 to 6

    · W is:

        - either SU with U being an optionally substituted alkyl, aryl or oside radical,

        - or $-\overset{\displaystyle \|}{\underset{\displaystyle Y^2}{C}}-Z$ with Y^2 being O or S, and

        Z being an optionally substituted alkyl, aryl or oside radical.

"Modified oligonucleotide" is understood here to mean modifications in the sugar part, in the base or alternatively in the phosphate linkage, or even in the anomeric configuration of the linkages. These X groups undergo an enzymatic cleavage of the Y_1/W bond by the enzymatic activation of intracellular enzymes according to a mechanism illustrated in Figure 1.

When U and Z are an alkyl, aryl or oside radical, the C_1 and C_7 alkyl radicals, benzyl and phenyl radicals and, as sugars, glucose, mannose or rhamnose are mentioned in particular.

Among these X groups, $-(CH_2)_n-S-S-U$ or

$-(CH_2)_n-O-\overset{\displaystyle \|}{\underset{\displaystyle Y_2}{C}}-Z$ are more particularly mentioned and more

particularly still $-(CH_2)_n\ O\ \overset{\displaystyle \|}{\underset{\displaystyle O}{C}}\ Z,\ -(CH_2)_n-S-S-U,\ -(CH_2)_n\ S\ \overset{\displaystyle \|}{\underset{\displaystyle O}{C}}\ Z$

where n = 1 or 2.

These phosphorothioate triester linkages are converted under the influence of intracellular enzymes either into phosphodiesters or into phosphorothioate diesters, as is shown by the decomposition studies on cellular extracts presented below and the mechanisms indicated in Figure 1.

Among the alkyls forming the groups U or Z, the lower alkyls optionally substituted by a group chosen

particular.

Thus, in a particular embodiment of the invention, X is $-(CH_2)_n-S-S-(CH_2)_{n^1}-X^1$ with n and n^1 being an integer from 1 to 4, preferably 2, and X^1 is H, OH, SH or NH$_2$, or X is $-(CH_2)_n-Y^1-CO-Z$, where n = 1 or 2 and Z = CH$_3$ or tBu. More particularly still X = tBu COOCH$_2$-, CH$_3$COSHCH$_2$CH$_2$- or CH$_3$COSCH$_2$-.

The electrically charged phosphorothioate diester oligomers are somewhat insensitive to intracellular nuclease degradation. This is why, in an advantageous embodiment according to the invention, the oligonucleotides will be formed by a chimeric oligomer comprising a central DNA or RNA sequence, the internucleotide linkages of which contain a $Y = \overset{|}{\underset{|}{P}} - SX$ bond, this central sequence being flanked in the 5' and 3' positions by DNA or RNA sequences modified so that they are resistant to nucleases and/or stabilize hybridization with a complementary strand.

This embodiment is particularly advantageous in the case of an anti-sense approach in which an oligonucleotide of the DNA type is used so as to form a DNA/RNA duplex which will thus be a substrate of RNAse H. These chimeric oligomers according to the invention combine all the advantageous properties of cellular penetration, resistance to intra- and extracellular enzymes and formation of duplex substrates of RNAse H.

In particular, compounds of formula I will be used in which R_1 and R_2 have sequences at the 5' and 3' ends with electrically neutral internucleotide linkages which are resistant to degradation by extra- and intracellular nucleases, such as linkages of the methylphosphonate type.

According to another embodiment, the oligonucleotides according to the invention are formed from a chimeric oligomer comprising a β-anomeric central DNA or RNA sequence, the internucleotide linkages of which are of the type Y = P-SX, this central sequence being flanked in the 5' and 3' positions by alpha-anomeric DNA or RNA

sequences.

In particular, an oligonucleotide according to the invention can be formed from a chimeric oligomer containing a central sequence of phosphorothioate triest-

5 er β-nucleoside linkage with internucleotide linkage of

the $Y - \overset{|}{\underset{|}{P}} - SX$ type surrounded by flanks formed from

sequences of phosphate diester form alpha-nucleoside linkages.

Generally speaking, different nucleotides can be

10 part of the formulation of oligonucleotides according to the invention. The oligonucleotides to which the present invention relates can be formed by a sequence of nucleotide bases including adenine (A), guanine (G), cytosine (C), thymine (T) and uracil (U), or the oligonucleotides

15 can likewise include rare nucleotides (inosine, I, or rI, for example) or modified nucleotides, either in the deoxyribo- series or in the ribo- series, interconnected by non-modified or modified phosphodiester bonds according to the present invention.

20   In particular, the oligonucleotides can include reactive nucleotides capable of establishing bonds with the sequence of the target molecule complementary to the oligonucleotide.

Thus, the oligonucleotides according to the

25 invention can carry reactive groups grafted onto the nucleotides, such as, for example psoralen groups, or other bridging agents or intercalating agents able to react with the sequence of the target molecule complementary to the oligonucleotide.

30   Likewise oligonucleotides form part of the invention, which are coupled to molecules allowing their intracellular penetration to be increased, and in particular lipophilic groups, polypeptides or proteins.

The oligonucleotides according to the invention

35 are prepared starting from an oligonucleotide having phosphothioate diester internucleotide linkages of the

type $Y - \overset{|}{\underset{|}{P}} - S^-$. A nucleophilic substitution reaction by the

sulfur atom of the $-P-S^-$ bond of said phosphorothioate diester linkages is carried out on an alkylating agent XL, thus leading to the formation of an oligonucleotide with phosphorothioate triester linkages $Y = \overset{|}{\underset{|}{P}} - SX_r$, L

5   being a leaving group such as halogen, ester or tosyl, and X being a bioreversible group according to the invention.

The phosphorothioate diester oligonucleotides with a $Y = \overset{|}{\underset{|}{P}} - S^-$ linkage are prepared by conventional

10   methods of synthesis, by a chemical or biochemical route, or by approaches calling for combinations of chemical synthesis and molecular biology techniques.

Among the methods described as conventional, various methods of chemical synthesis of oligonucleotides

15   have been developed and are well known to the specialists working in these fields. For example, one method consists in using a solid support called CPG (controlled pore glass) to which the first nucleoside is fixed covalently by a spacer through its 3'OH end. The 5'OH end of the

20   nucleoside is protected by an acid-label di-p-methoxy-trityl group. This approach, using phosphite triester chemistry, and in which deoxynucleoside 3'-phosphor-amidites are used as synthons is called the phosphor-amidite method. This approach is most used at present and

25   has the advantage of being entirely automatic.

Another approach used for the synthesis of oligonucleotides is that of phosphonate chemistry. This approach starts with the condensation of a deoxy-nucleoside 3'-H-phosphonate with a deoxynucleoside

30   coupled to a glass or silica support. Successive conden-sation cycles lead to the synthesis of oligonucleotide H-phosphonates. These oligonucleotides are oxidized in one step to give the phosphodiesters.

Using one or the other of these techniques, or

35   any other sequential procedure allowing the synthesis of polynucleotide chains of determined sequence in advance, oligonucleotides having the desired starting structure

are obtained.

Detailed syntheses of phosphorothioate diester oligonucleotides have been described, for example, in J. Am. Chem. Soc. 106: 6077-6079 (1984) and Nucleic Acids
5   Res. 14: 5399-5407 (1986).

The oligonucleotides can be used as various diagnostic, cosmetic or pharmacological compositions at variable concentrations and with the appropriate excipients according to the applications.
10   Other characteristics and advantages of the present invention will appear in the light of the examples which follow.

Figure 1 shows the mechanisms of intracellular decomposition of bioreversible groups.

15   I - Examples 1 to 3: Synthesis of phosphorothioate triester dinucleosides 4a, 4b, and 4c and evaluation of their stability in biological medium

I-1 - Synthesis of the compounds 4a, 4b and 4c

General conditions:
20   Thin-layer chromatography (TLC) is carried out on Merck 60 F_{254} silica gel sheets. The products are detected with a UV lamp (254 nm) and visualized by heating after spraying with 10% sulfuric acid in 95° ethanol.
Chromatography on a silica gel column is carried out with
25   silica Kieselgel 60 (40 μm-63 μm).

Proton NMR spectra are recorded at ambient temperature on a Brüker AC 250 apparatus. The samples are solubilized in DMSO-d_6. The chemical shifts (δ) are expressed in ppm with reference to the signal of DMSO-d_5
30   fixed at 2.49 ppm, taken as internal reference. The coupling constants are given in Hertz. The multiplicity of the signals observed is indicated by a small letter:

m: multiplet; pq: pseudo-quartet, t: triplet; d: doublet; s: singlet; sl: broad singlet
35   The nucleoside having its 5'OH function free is represented by N_1 and the nucleoside having its 3'OH function

free is represented by N_2.

    The phosphorus NMR spectra are recorded at ambient temperature on a Brüker WP 200 SY apparatus with proton decoupling. The samples are solubilized in CD_3CN

5   or in DMSO-d_6. The chemical shifts are expressed in ppm in relation to 66% H_3PO_4 taken as external reference.

    The mass spectra are carried out on a JEOL JMS DX 300 apparatus by the FAB ionization method in positive mode with different matrices: glycerol (G), glycerol/-

10 thioglycerol (GT) or 3-nitrobenzyl alcohol (NBA).

    The acetonitrile was distilled after heating to reflux for one night over calcium hydride and is kept over molecular sieve (4A).

    The alkylating agents were synthesized according

15 to processes described in the literature:

-     pivaloyloxymethyl iodide: European Patent Appl. No. 843080672

-     bromoethylacetyl sulfide: P. Nylen, A. Olsen, *Svensk Kem. Tid.*, <u>53</u>, 274 (1941)

20 -   bromomethylacetyl sulfide: G.K. Farrington, A. Kumar, F.C. Wedler, *Oppi briefs*, <u>21</u>, 390 (1989).

    The phosphorothioate diester dinucleosides were synthesized according to conventional methods described in the literature, using H-phosphonate chemistry

25 (J. Stawinski, M. Thelin, *Nucleosides and Nucleotides*, <u>9</u>, 129 (1990), P.J. Garegg, T. Regberg, J. Stawinsky, R. Strömberg, *Nucleosides and Nucleotides*, <u>6</u>, 655 (1987)).

    Aqueous solutions of 1 M and 0.5 M triethyl-

30 ammonium hydrogen carbonate (TEAB) were used to neutralize the reaction media and to carry out the extractions.

    Before lyophilization, the solutions are filtered on a Millex HV-4 filter (Millipore, 0.45 μm).

Synthesis:
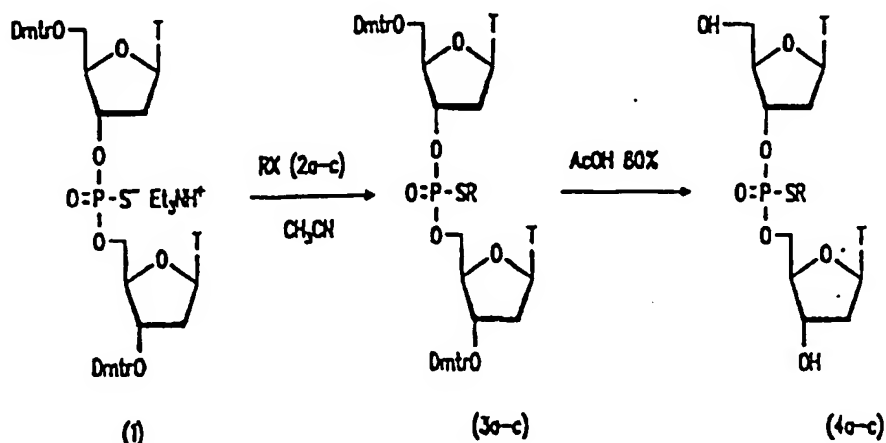
a: R = tBuCOOCH$_2$- ; b, d: R = CH$_3$COSCH$_2$CH$_2$- ; c: R = CH$_3$COSCH$_2$-

X = I (a, c et d); Br (b)

Example 1

    O-[5'-O-(4,4'-Dimethoxytrityl)thymidin-3'yl]
O-[3'-O-(4,4'-dimethoxytrityl)thymidin-5'-yl]

5   S-(pivaloyloxymethyl) phosphorothioate (3a):

    Pivaloyloxymethyl iodide (2a) (484 mg, 2 mmol) is added to a solution of phosphorothioate diester dinucleoside (1) (254 mg; 0.2 mmol) in anhydrous acetonitrile (10 ml). The reaction is stirred at ambient temperature

10   for 30 minutes, then the reaction medium is neutralized by addition of an aqueous solution of 1 M TEAB (2 ml) and left for 5 minutes with stirring. The reaction mixture is then poured onto an aqueous solution of 0.5 M TEAB (50 ml) and extracted with dichloromethane (3 × 30 ml).

15   The organic phases are collected, washed with water (50 ml), dried over sodium sulfate, filtered and then evaporated to dryness. The residue is then chromatographed on a silica gel column using a gradient of methanol (0 to 2%) in dichloromethane as eluent. The

20   fractions containing (3a) are combined and concentrated under reduced pressure, leading to a white solid residue (220 mg; 86%) which is carefully dried for use in the following step.

    R_f = 0.67 (CH$_2$Cl$_2$/MeOH: 9/1).

O-(Thymidin-3'-yl)   O-(thymidin-5'-yl)
S-(pivaloyloxymethyl) phosphorothioate (4a):

The totally protected dimeric phosphorothioate
triester (3a) is dissolved in a mixture of acetic acid/
water/methanol (8:1:1, v:v:v) (10 ml). The reaction is
left with stirring at ambient temperature for 5 hours.
The reaction medium is then evaporated and the residue is
coevaporated several times with water and toluene. The
residue is then chromatographed on a silica gel column
using a gradient of methanol (0 to 10%) in dichloro-
methane as eluent. The fractions containing the product
(4a) are collected, concentrated under reduced pressure,
and then the residue is coevaporated several times with
dioxane and lyophilized in dioxane.

(4a) is obtained in the form of a white powder (98 mg,
85%).

$R_f = 0.2$ (CH$_2$Cl$_2$/MeOH: 9/1)

MS: FAB>0 (NBA): 677: (M+H)$^+$; 551: (M-B)$^+$; 424:
(M-(2B+2H))$^+$

^{31}P NMR (CD$_3$CN): δ = 27.21 and 27.31 ppm (2 diastereo-
isomers)

^{1}H NMR (DMSO-d$_6$): δ = 11.34 (sl. 2H, 2NH); 7.68 and 7.48
(2m, 2H, 2H$_6$): 6.21 (m, 2H, 2H$_{1'}$); 5.48 (d, 1H, OH$_{3'}$,
J=4.4 Hz); 5.41 (d, 2H, SCH$_2$O, J=20 Hz); 5.26 (t, 1H,
OH$_{5'}$, J=5.1 Hz), 5.10 (m, 1H, H$_{3'}$ (N$_1$)); 4.26 (m, 3H, H$_{3'}$
(N$_2$), H$_{5'}$ and H$_{5''}$ (N$_2$)); 4.12 (m, 1H, H$_{4'}$ (N$_1$)); 3.96 (m, 1H,
H$_{4'}$ (N$_2$)); 3.61 (m, 2H, H$_{5'}$ and H$_{5''}$ (N$_1$)); 2.38 (m, 2H, H$_{2'}$
and H$_{2''}$ (N$_1$)); 2.14 (m, 2H, H$_{2'}$ and H$_{2''}$ (N$_2$)); 1.79 and 1.77
(2s, 2CH$_3$ (N$_1$ and N$_2$)); 1.13 (d, 9H, (CH$_3$)$_3$C) ppm.

Example 2

O-[5'-O-(4,4'-Dimethoxytrityl)thymidin-3'-yl]
O-[3'-O-(4,4'-dimethoxytrityl)thymidin-5'-yl] S-(acetyl-
thioethyl) phosphorothioate (3b):

Bromoethylacetyl sulfide (2b) (360 mg; 1.97 mmol)
is added to a solution of phosphorothioate diester
dinucleoside (1) (50 mg; 0.0394 mmol) in anhydrous
acetonitrile (5 ml). The reaction mixture is stirred at
ambient temperature for 5 days, then it is neutralized by

addition of an aqueous solution of 1 M TEAB (1 ml) and left with stirrring for 5 minutes. The reaction mixture is then poured onto an aqueous solution of 0.5 M TEAB (25 ml) and extracted with dichloromethane (3 × 15 ml).

5 The organic phases are collected, washed with water (25 ml), dried over sodium sulfate, filtered and then evaporated to dryness. The residue is then chromatographed on a column of silica gel using a gradient of methanol (0 to 4%) in dichloromethane as eluent. (3b) is

10 obtained in the form of a white solid (27 mg, 55%).

R_f = 0.75 (CH_2Cl_2/MeOH: 9/1).

O-(Thymidin-3'-yl) O-(thymidin-5'-yl) S-(acetyl-thioethyl) phosphorothioate (4b):

The totally protected phosphorothioate triester

15 dinucleoside (3b) is dissolved in an acetic acid/water/ methanol mixture (8:1:1, v:v:v) (5 ml) and left with stirring all night. The following day, the reaction mixture is evaporated and the residue is coevaporated several times with water and then with toluene. The solid

20 residue is then chromatographed on a column of silica gel using a gradient of methanol (0 to 10%) in dichloromethane as eluent. The fractions containing the detritylated phosphorothioate triester dinucleoside (4b) are collected and evaporated under reduced pressure, then the

25 residue is coevaporated with dioxane and lyophilized in dioxane. (4b) is obtained in the form of a white powder (12 mg, 86%).

R_f = 0.35 (CH_2Cl_2/MeOH: 9/1)

MS: FAB>0 (GT): 665: (M+H)$^+$

30 ^{31}P NMR (DMSO-d_6): δ = 28.09 and 28.22 ppm

^{1}H NMR (DMSO-d_6): δ = 11.34 (2s, 2H, 2NH); 7.68 and 7.48 (2d, 2H, 2H$_6$); 6.20 (t, 2H, 2H$_1$.); 5.48 (d, 1H, OH$_3$.), 5.26 (pq, 1H, OH$_5$.), 5.09 (m, 1H, H$_3$. (N$_1$)), 4.26 (m, 3H, H$_3$. (N$_2$), H$_5$. and H$_5$. (N2)); 4.10 (m, 1H, H$_4$. (N$_1$)); 3.95 (m,

35 1H, H$_4$. (N$_2$)); 3.61 (m, 2H, H$_5$. and H$_5$. (N$_1$)); 3.13 and·3.02 (2m, 4H, SCH$_2$CH$_2$S); 2.29 (m, 2H, H$_2$. and H$_2$. (N$_1$)); 2.33 (d, 3H, CH$_3$CO); 2.13 (m, 2H, H$_2$. and H$_2$. (N$_2$)); 1.77 and 1.78 (2s, 6H, 2CH$_3$ (N$_1$ and N$_2$)) ppm.

Example 3

O-[5'-O-(4,4'-Dimethoxytrityl)thymidin-3'-yl]
O-[3'-O-(4,4'-dimethoxytrityl)thymidin-5'-yl] S-(acetyl-
thiomethyl) phosphorothioate (3c):

5   Iodomethylacetyl sulfide (98 mg; 0.457 mmol) is
added to a solution of phosphorothioate diester dinucleo-
side (1) (58 mg; 0.0457 mmol) in anhydrous acetonitrile
(5 ml). The reaction is left with stirring all night at
ambient temperature, then the reaction mixture is
10   neutralized by addition of a 1 M aqueous solution of TEAB
(1 ml) and left with stirring for several minutes. The
reaction mixture is then poured onto a 0.5 M aqueous
solution of TEAB (25 ml) and extracted with dichloro-
methane (3 × 15 ml). The organic phases are collected,
15   washed with water (25 ml), dried over sodium sulfate,
filtered and then evaporated to dryness. The residue is
then chromatographed on a column of silica gel using a
gradient of methanol (0 to 3%) in dichloromethane as
eluent. (3c) is obtained in the form of a white solid
20   (46 mg, 80%).

R_f = 0.55 (CH$_2$Cl$_2$/MeOH: 9/1).

O-(Thymidin-3'-yl) O-(thymidin-5'-yl) S-(acetyl-
thiomethyl) phosphorothioate (4c):

          The totally protected phosphorothioate dinucleo-
25   side (3c) is dissolved in an acetic acid/water/methanol
mixture (8:1:1, v:v:v) (5 ml) and left with stirring at
ambient temperature all night. The following day, the
reaction mixture is evaporated and coevaporated several
times with water and with toluene. The residue is then
30   chromatographed on a column of silica gel using a
gradient of methanol (0 to 10%) as eluent. The fractions
containing the detritylated phosphorothioate triester
dinucleoside (4c) are collected and concentrated under
reduced pressure. The residue is coevaporated with
35   dioxane and then lyophilized in dioxane. (4c) is obtained
in the form of a white powder (20 mg, 85%).

R_f = 0.62 (CH$_2$Cl$_2$/MeOH: 8/2)

MS: FAB>0 (GT): 651 (M+H)*

^{31}P NMR (CD$_3$CN): δ = 27.22 and 27.44 ppm (2 diastereo-

isomers)

^{1}H NMR (DMSO-d_6): δ = 11.34 (d, 2H, 2 NH); 7.70 and 7.49 (2d, 2H, 2H$_6$); 6.22 (m, 2H, 2H$_{1'}$); 5.48 (d, 1H, OH$_{3'}$); 5.37 (m, 1H, OH$_{5'}$); 5.08 (m, 1H, H$_{3'}$ (N$_1$)); 4.29 (m, 5H, H$_{3'}$ (N$_2$), H$_{5'}$ and H$_{5'}$ (N$_2$), SCH$_2$S); 4.13 (m, 1H, H$_{4'}$ (N$_1$)); 3.97 (m, 1H, H$_{4'}$ (N$_2$)); 3.62 (m, 2H, H$_{5'}$ and H$_{5'}$ (N$_1$)), 2.39 (m, 5H, H$_{2'}$ and H$_{2'}$ (N$_1$), CH$_3$CO), 2.15 (m, 2H, H$_{2'}$ and H$_{2'}$ (N$_2$)); 1.78 (s, 6H, 2CH$_3$ (N$_1$ and N$_2$)).

I-2 - Stability studies on dimeric phosphorothioate triesters **4a**, **4b** and **4c**

General conditions:

· *Method*

The stability of the dimers (4a), (4b) and (4c) in different biological media were studied according to an HPLC technique perfected in the laboratory (A. Pompon, I. Lefebvre, J.L. Imbach, *Biochemical Pharmacology* **43**, 1769 (1992)), which does not require any preliminary handling of the sample and allows its direct injection; a precolumn, which allows proteins to be eliminated, filled with a new material ISRP (Internal Surface Reverse Phase) is combined with a high resolution column (inverse phase) allowing chromatographic analysis.

· **Equipment:**

The chromatograph (Waters-Millipore) is composed of:

      - an M 680 programmer

      - two M 510 pumps

      - a WISP 712 automatic injector

      - an M 990 UV diode array detector

      - an NEC APC IV microcomputer

      - a Waters 990 printer

      - a model 7010 6-way valve (Rheodyne).

The columns are supplied by SFCC Shandon:

      - ISRP precolumn (Ultrabiosep C$_{18}$, 10 μm, 4.6 mm × 10 mm)

      - Inverse phase analytical column (Nucleosil C18, 5 μm, 4.6 mm × 100 mm)

The analytical column is thermostatted at 30°C.

The analysis of the results is carried out on the

EUREKA software.

· *Chemical products:*

Distilled water is purified on a MilliQ system (Waters-Millipore),

5   Acetonitrile is of HPLC-far UV quality (Fisons),

Ammonium acetate is of "analytical" quality (MERCK),

The culture media are composed of 90% of RPMI 1640 and 10% of heat-inactivated fetal calf serum (GIBCO),

The cellular extracts were kindly supplied by

10   Miss A.-M. Aubertin (University of Strasbourg I). They are prepared in the following way: CEM cells in the exponential growth phase are separated from the culture medium by centrifugation (10^4 g, 4 min, 4°). The residue (approximately 100 μl, 5×10^7 cells) is dissolved in 2 ml

15   of buffer (Tris-HCl 140 mM, pH = 7.4) and sonicated. The lysate is centrifuged (10^5 g, 1h, 4°) to eliminate membranes, organelles and chromatin.

Two types of *eluents* were utilized for the HPLC:

       - eluent A: 0.1 M ammonium acetate buffer, pH = 5.9,

20   - eluent B: 0.1 M ammonium acetate buffer, 50% acetonitrile, pH = 5.9.

· *Preparation of the samples:*

       A 10^{-2} M solution of the compound to be studied is prepared in DMSO.

25   This solution is diluted with water to give a parent solution of concentration 5×10^{-4} M.

       a) - Study of stability in culture medium:

       100 μl of parent solution of the compound to be studied are added to 900 μl of culture medium which has

30   previously been filtered on a Millex GV sterile filter (Millipore, 0.22 μm). After mixing, fractions (100 μl) are distributed in sterile Eppendorf tubes. These tubes are placed in an oven at 37°C and removed as a function of the kinetic development. The samples are immediately

35   analyzed by HPLC (volume injected: 80 μl) or preserved at -25°C with a view to subsequent analysis.

       b) - Study of stability in cellular extract:

       100 μl of parent solution of the compound to be studied are added to 900 μl of cellular extract which has

previously been filtered on a Millex GV sterile filter (Millipore, 0.22 μm). After mixing, fractions (100 μl) are distributed in Eppendorf tubes. These tubes are placed in an oven at 37°C and removed as a function of

5 the kinetic development. The samples are immediately analyzed by HPLC.

Results:

The results regarding the stability studies of the three dimers (4a), (4b) and (4c) are collected in the

10 table below:

C_0 = 5 × 10^{-5} M

$t_{1/2}$ = time at the end of which half of the starting product has decomposed

PO$^-$ = phosphodiester dinucleoside

15 PS$^-$ = phosphorothioate diester dinucleoside

% PO$^-$, % PS$^-$ = molar fractions of phosphodiester dinucleoside and of phosphorothioate diester dinucleoside, expressed in relation to the initial quantity of phosphorothioate triester dinucleoside.

20 The percentage of PS$^-$ corresponds to the quantity of phosphorothioate diester dinucleoside formed after complete decomposition of the starting phosphorothioate triester dinucleoside (considering that the phosphorothioate diester dinucleoside is stable under the con-

25 ditions used and accumulates).

The percentage of PO$^-$ is calculated by the difference (100 - % PS$^-$ formed) because the phosphodiester dinucleoside decomposes rapidly from its formation.

	RPMI + 10% serum			cellular extract		
	$t_{1/2}$	% PO$^-$	% PS$^-$	$t_{1/2}$	% PO$^-$	% PS$^-$
(4a)	6 h	80	20	40 min	/	100
(4b)	7 h	50	50	10 min	/	100
(4c)	1 h	20	80	5-10 min	/	100

It follows from the stability study of the compounds <u>4a-c</u> presented above that these are rapidly and selectively converted in cellular extract into their corresponding phosphorothioate diester, which is in
5   agreement with the original hypothesis.

II - Examples 4 to 6: Synthesis of oligonucleotides containing phosphorothioate triester bonds by post-synthesis alkylation of phosphorothioate diester bonds. Evaluation of their stability in biological media

10   II-1 - Synthesis of oligonucleotides <u>5c</u>, <u>6b</u> and <u>7b</u> containing phosphorothioate triester bonds
<u>General conditions:</u>
        The oligonucleotides containing phosphorothioate diester bonds were synthesized on a solid phase in an
15   Applied Biosystems model 381 A DNA synthesizer, on support columns corresponding to one μmole of grafted nucleoside.
        The purification and analysis of the oligonucleotides <u>5c</u>, <u>6b</u> and <u>7b</u> were carried out by HPLC using
20   a chromatographic system composed of Waters-Millipore equipment:
- an M 680 programmer
- an NEC APC IV computer
- a Waters 990 printer
25   - two M 510 pumps
- a U$_6$K injector
- a UV diode array detector
        The columns and the precolumns are supplied by SFCC-Shandon:
30   - in the case of preparative HPLC, a Nucleosil $C_{18}N$ 525 column (10 mm × 250 mm) of particle size 5 μm was used, protected by a Nucleosil $C_{18}PSF$-25 precolumn of particle size 5 μm,
              - in the case of analytical HPLC, a Nucleosil
35     $C_{18}N$ 125 column (4.6 mm × 150 mm) of particle size 5 μm protected by a Nucleosil $C_{18}PSF$-25 precolumn of particle size 5 μm used.

After purification, the oligonucleotides are filtered on a Millex HV-13 filter (Millipore, 0.45 μm) and submitted to several successive lyophilizations in water.

5   Following alkylation reactions of the oligo-nucleotides and of purification of the alkylated oligo-nucleotides 5c, 6b and 7b were carried out by HPLC on a Waters-Millipore chromatograph composed of:

- a 600 E programmer
10   - a 600 pumping system
- a U$_6$K manual injector
- a 486 UV detector
- a Powermate SX Plus microcomputer.

Follwing of alkylation reactions was carried out 15 on a Nucleosil C$_{18}$ 5 μm column (4.6 mm × 150 mm) protected by a Nucleosil C$_{18}$ 5 μm precolumn (SFCC - Shandon).

The purification of the oligonucleotides 5c, 6b and 7b was carried out on a RADIAL-PAK C$_{18}$ 10 μm cartridge to support the RCM column (8 × 10), protected by a 20 GUARD-PAK Resolve C$_{18}$ precolumn (Waters-Millipore).

The desalting of the samples was carried out on a C$_{18}$ SEP-PAK cartridge (Waters-Millipore).

Before lyophilization, the solutions are filtered on a Millex HV-13 filter (Millipore, 0.45 μm).

25   II-1-1 - Example 4: Synthesis of the chimeric dodecamer 5c containing a phosphorothioate triester central window and methylphosphonate flanks
a) - Synthesis of a dodecamer containing a phosphoro-thioate diester central window and methylphosphonate
30 flanks
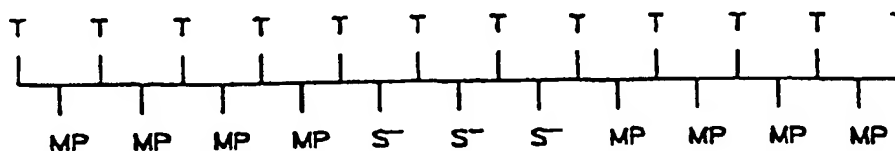
The standard elongation cycle proposed for the synthesis of oligonucleotides was used, using the methyl-phosphonamidite synthon for the flanks and the mixture

I_2/THF/water/pyridine as oxidation agent, and the cyano-
ethyl phosphoramidite synthon and the Beaucage reagent as
sulfuration   agent   (R.P. Iyer,   W. Egan,   J.B. Regan,
S.L. Beaucage, *J. Am. Chem. Soc.*, **112**, 1253 (1990)) for

5   the central part (3 incorporation cycles).

The detachment of the oligonucleotide from the
support and its deprotection were carried out according
to the procedure conventionally described for methyl-
phosphonates   (P.S. Miller,   M.P. Reddy,   A. Murakami,

10   K.R. Blake, S.B. Lin, C.H. Agris, *Biochemistry*, **25**, 5092
(1986)) using a solution of ethylenediamine in absolute
ethanol (1:1, v:v).

The synthesis on the one micromole scale led us
to a rough synthesis figure of 75 absorption units at

15   260 nm (A_{260} units).

After   purification   by   preparative   HPLC,   we
obtained 37 A_{260} units of chimeric oligonucleotide of a
spectrophotometric purity of 100% (determined by HPLC
analysis).

20   b) - Alkylation of the phosphorothioate diester bonds.
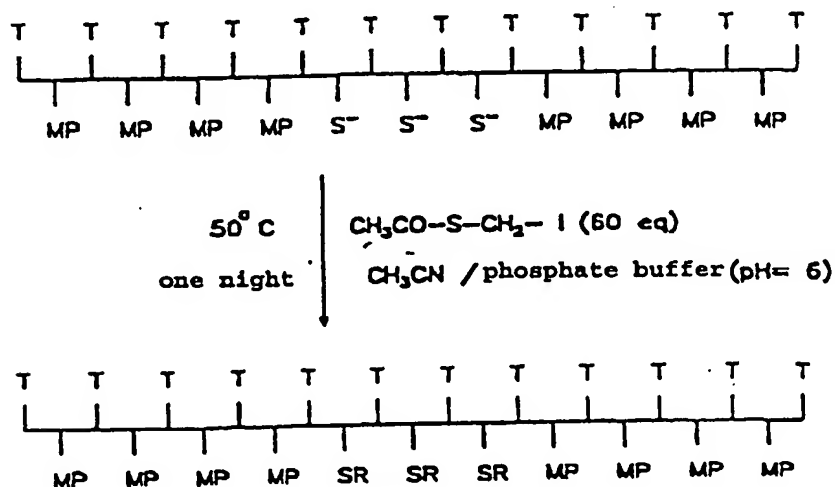
The following are introduced successively into an
Eppendorf:
    - 120 μl of acetonitrile
    - 135 μl of 20 mM phosphate buffer (pH = 6.15)

25   - 15 μl of iodomethylacetyl sulfide in 0.92 M
solution in acetonitrile

- 30 μl of 7.66 mM solution of oligonucleotide in water (being approximately 22 A_{260} units)

The Eppendorf is placed in a dry bath previously thermostatted at 50°C.

Samples of 5 μl are taken at different reaction times and are analyzed by HPLC in order to estimate the progress of the reaction.

After 16 hours of reaction, the presence of the starting oligonucleotide is no longer detected (t_r = 12.99 min) and the totally alkylated oligonucleotide (t_r = 17.53 min) is purified by HPLC.

The oligonucleotide is then desalted, before being lyophilized in a water/dioxane mixture (50:50, v:v) and analyzed again by HPLC.

10 A_{260} units of totally alkylated oligonucleotide of a spectrophotometric purity of 97% were obtained (determined by HPLC).

II-1-2 - Example 5: Synthesis of the entirely phosphoro-thioate triester dodecamer 6b

a) - Synthesis of a phosphorothioate diester dodecamer d($C_{P(S)}A_{P(S)}C_{P(S)}C_{P(S)}C_{P(S)}A_{P(S)}A_{P(S)}T_{P(S)}T_{P(S)}C_{P(S)}T_{P(S)}G$)

The standard cycle of elongation proposed for the synthesis of oligonucleotides using cyanoethyl phosphor-amidite synthons of the four bases suitably protected and replacing the oxidation step with I_2/THF/water/pyridine by a sulfuration step with the Beaucage reagent was used.

The detachment of the oligonucleotide from the support and its deprotection were carried out by treat-ment with concentrated ammonia (32%) overnight at 55°C.

b) - Alkylation of the phosphorothioate diester bonds

Phosphate buffer (pH = 6.15, 123 μl), aceto-nitrile (120 μl) and a 1 M solution of iodoethylacetyl sulfide (2d) (55 μl) are added successively to a solution of 6.4 mM phosphorothioate diester oligonucleotide, d($C_{P(S)}A_{P(S)}C_{P(S)}C_{P(S)}C_{P(S)}A_{P(S)}A_{P(S)}T_{P(S)}T_{P(S)}C_{P(S)}T_{P(S)}G$), in water (42 μl). The mixture is maintained at 50°C for 48 hours. After evaporation, the residue is purified by HPLC. The fraction containing the completely alkylated phosphoro-

thioate dodecamer 6b is desalted and the evaporation residue taken up again in a water/dioxane mixture (50:50, v:v, 1 ml) is lyophilized.

Yield 35%. Reference (2d) refers to the nomenclature used in the synthesis scheme of the paragraph I-1.

II-1-3 - Example 6: Synthesis of the chimeric dodecamer 7b containing a central window formed of phosphorothioate triester β-nucleosides and of flanks formed of phosphate diester α-nucleosides.

a) - Synthesis of the dodecamer containing a β-phosphorothioate diester window and of d[α-(TCTT)3'$\rightarrow$3'β-($T_{P(s)}T_{P(s)}C_{P(s)}$C)5'$\rightarrow$5'α-(CTCT)] α-phosphate diester flanks

The standard cycle of elongation proposed for the synthesis of oligonucleotides using α-anomeric cyanoethyl phosphoramidite synthons suitably protected on the bases for the flanks and the mixture I_2/THF/water/pyridine as oxidizing agent, and the β-anomeric cyanoethyl phosphoramidite synthons suitably protected on the bases for the central part and the Beaucage reagent as sulfuration agent were used.

b) - Alkylation of the phosphorothioate diester bonds

Phosphate buffer (pH = 6.15, 145 μl) and a solution of 1 M iodoethylacetyl sulfide (2d) (55 μl) are added successively to an 11 mM solution of d[α-(TCTT)3'$\rightarrow$3'β-($T_{P(s)}T_{P(s)}C_{P(s)}$C)5'$\rightarrow$5'α-(CTCT)] oligonucleotide in water (20 μl). The mixture is maintained at 50°C for 48 hours. After evaporation, the residue is purified by HPLC. The fraction containing the dodecanucleotide (7d) alkylated on the three central phosphorothioate bonds is desalted and then lyophilized. Yield 47%.

II-2 - Study of stability of the chimeric oligonucleotides 5c, 6b and 7b in biological media.
General conditions:

The general conditions are the same as those used for the study of stability of the phosphorothioate triester dimers, as far as the method, the equipment and

the chemical products are concerned.

· Preparation of the samples:

A parent solution of oligonucleotide is prepared in dioxane (10 A_{260} units of oligonucleotide in 200 μl of dioxane).

a) - Study of stability in culture medium:

30 μl of parent solution of oligonucleotide (being approximately 1.5 A_{260} units) are removed, which are added to 1470 μl of culture medium which has previously been filtered on a sterile Millex GV filter (Millipore, 0.22 μm). After mixing, fractions (100 μl) are distributed in sterile Eppendorf tubes. These tubes are placed in an oven at 37°C and removed as a function of the kinetic development. The samples are immediately analyzed by HPLC (volume injected 80 μl) or preserved at -25°C with a view to subsequent analysis.

b) - Study of stability in cellular extract:

10 μl of oligonucleotide parent solution are added to 990 μl of cellular extract which have previously been filtered on a sterile Millex GV filter (Millipore, 0.22 μm). After mixing, fractions (100 μl) are distributed in sterile Eppendorf tubes, placed in an oven at 37°C, sampled at different times and immediately analyzed by HPLC.

Results:

The results regarding the stability studies of the three oligonucleotides 5c, 6b and 7b are collected in the table below.

oligo-nucleotide studied	RPMI + 10% serum		cellular extract
	$t_{1/2}$ of disappearance of the starting oligonucleotide	$t_{1/2}$ of disappearance of the starting oligonucleotide	$t_{1/2}$ of formation of the totally deprotected oligonucleotide
5c	40 min	< 5 min	20 min
6b	55 min	< 2 min	25 min
7b	35 min	< 2 min	20 min

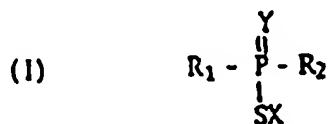All of the data regarding the compounds 5c, 6b

and 7b shows without ambiguity that it is possible to protect internucleotide phosphorothioate functions selectively by bioreversible groups. In addition, it is indeed confirmed in cellular extracts that such phos-

5   phorothioate triester oligonucleotides are rapidly deprotected.

     Figure 1 shows the mechanism of decomposition of bioreversible groups according to the invention.

CLAIMS

1.   Phosphorothioate triester oligonucleotide, characterized in that it comprises internucleotide linkages which have a $P-S^-$ bond protected by a bio-reversible group (X) in intracellular media.

2.   Oligonucleotide according to Claim 1, characterized in that it corresponds to the following general formula

$$(I) \qquad R_1 - \overset{\overset{\displaystyle Y}{\|}}{\underset{\displaystyle SX}{P}} - R_2$$

in which:

- Y is O or S
- R_1 and R_2 are respectively a residue in the 3'-O and 5'-O positions of a nucleoside or of an oligonucleotide, the internucleotide linkage of which is natural or modified, the linkage of which is especially of the phosphorothioate triester type of formula

$$Y = \overset{\displaystyle |}{\underset{\displaystyle |}{P}} - SX$$
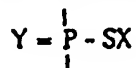
- X is a $-(CH_2)_n-Y^1-W$ radical or
  - Y^1 is S or O
  - n varies from 1 to 6
  - W is:
      - either $S\underline{U}$ with U being an optionally substituted alkyl, aryl or oside radical,
      - or $-\overset{\overset{\displaystyle }{\underset{\displaystyle Y^2}{\|}}}{C} - Z$ with Y^2 being O or S, and

          Z being an optionally substituted alkyl, aryl or oside radical.

3.   Oligonucleotide according to Claim 2, characterized in that X is $-(CH_2)_n - S - S - U$ or $-(CH_2)_n - O - \overset{\overset{\displaystyle }{\underset{\displaystyle Y_2}{\|}}}{C} - Z$

4.   Oligonucleotide according to Claim 2 or 3,

characterized in that X is. $-(CH_2)_n O \underset{\underset{O}{\overset{\|}{C}}}{} Z$, $-(CH_2)_n$-S-S-U, $-(CH_2)_n \underset{\underset{O}{\overset{\|}{S}C}}{} Z$,

where n = 1 or 2.

5.   Oligonucleotide according to one of Claims 2 to 4, characterized in that U and Z are a lower alkyl, optionally substituted by one or more groups chosen from amongst OH, SH and NH_2.

6.   Oligonucleotide according to Claim 5, characterized in that X is $-(CH_2)_n$-S-S-$(CH_2)_{n^1}$-X^1 with n and n^1 being an integer from 1 to 4, preferably 2, and X^1 is H, OH, SH or NH_2, or X is $-(CH_2)_n$-Y^1-CO-Z, where Z = CH_3 or tBu.

7.   Oligonucleotide according to one of Claims 1 to 6, characterized in that it is formed of a chimeric oligomer comprising a central DNA or RNA sequence, the internucleotide linkages of which comprise a $Y = \overset{\mid}{\underset{\mid}{P}} - SX$, bond, this central sequence being flanked in the 5' and 3' positions by DNA or RNA sequences modified so that they are resistant to nucleases and/or stabilize hybridization with a complementary strand.

8.   Oligonucleotide according to one of Claims 2 to 7, characterized in that R_1 and R2 [sic] have electrically neutral sequences at the 5' and 3' ends respectively, which are resistant to degradation by exonucleases.

9.   Oligonucleotide according to Claim 8, characterized in that the internucleotide linkages at the 5' and 3' ends respectively of R_1 and R_2 are of the methylphosphonate type.

10.   Oligonucleotide according to one of Claims 1 to 7, characterized in that it is formed from a chimeric oligomer comprising a β-anomeric central DNA or RNA sequence, the internucleotide linkages of which are of the $Y = \overset{\mid}{\underset{\mid}{P}} - SX$, type, this central sequence being flanked in the 5' and 3' positions by α-anomeric DNA or RNA

11.   Oligonucleotide according to Claim 10, charac-
terized in that it is formed from a chimeric oligomer
containing a surrounding central sequence of phosphoro-
thioate triester β-nucleosides with internucleotide

5   linkage of the $Y = \overset{|}{\underset{|}{P}} - SX$ type surrounded by flanks formed

from phosphate diester alpha-nucleoside linkages.

12.   Method of preparation of an oligonucleotide
according to one of Claims 1 to 11, characterized in that
a nucleophilic substitution reaction by the sulfur atom

10   of the -P-S bond of a phosphorothioate diester oligo-
nucleotide is carried out on an alkylating agent XL, thus

leading to the formation of $Y = \overset{|}{\underset{|}{P}} - SX$ phosphorothioate

triester internucleotide linkages, L being a leaving
group such as halogen, ester or tosyl, and X being a

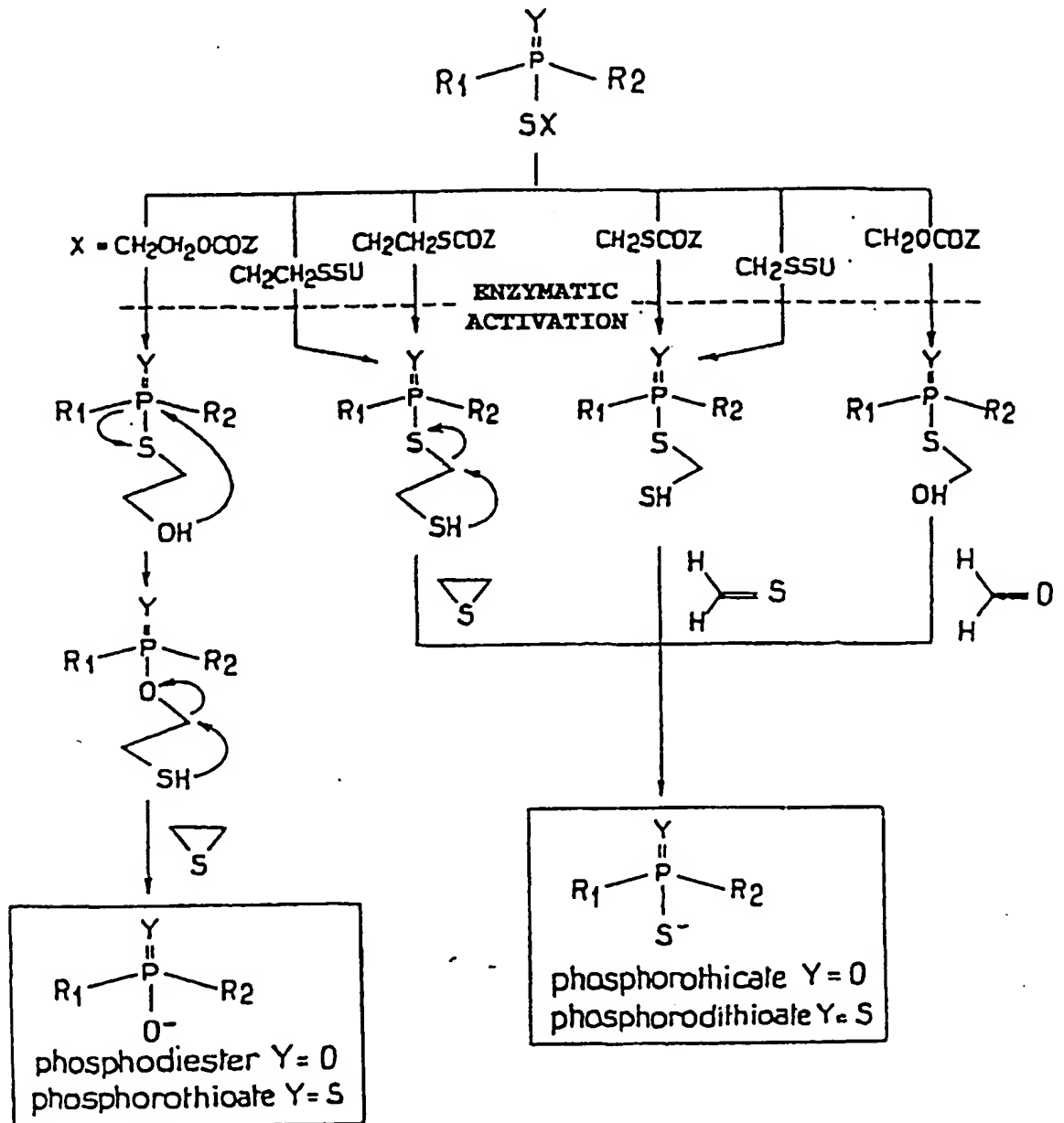15   bioreversible group such as defined in Claims 1 to 11.

FIG.1